# Increased Prostaglandin E_2 Has a Positive Correlation with Plasma Calcium during Goldfish Reproduction

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https://doi.org/10.5109/1434389

出版情報：九州大学大学院農学研究院紀要. 59 (1), pp.97-101, 2014-02-28. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **59** (1), 97–101 (2014)

# Increased Prostaglandin $E_2$ Has a Positive Correlation with Plasma Calcium during Goldfish Reproduction

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(*Received October 24, 2013 and accepted November 11, 2013*)

We recently demonstrated that prostaglandin $E_2$ ($PG_2$) increases osteoclastic activity and induces bone resorption in both *in vitro* and *in vivo* experiments using goldfish. In the fish reproductive period, the plasma calcium (Ca) level in female teleosts increases remarkably to make vitellogenin, which is a major component of egg protein and a Ca–binding protein. In this period, however, there is no reported relationship between $PGE_2$ and Ca metabolism in fish. To clarify the Ca metabolism in fish reproduction, we examined plasma $PGE_2$ and Ca levels and measured tartrate–resistant acid phosphatase (TRAP) activities as an indicator of osteoclastic activity in goldfish. Plasma $PGE_2$ levels in the reproductive stage significantly increased as compared with those in non–reproductive stages. Also, both plasma Ca and TRAP increased in the reproductive stage. Significant positive correlations were recognized between plasma Ca and the gonad somatic index ($r$=0.81, $p$<0.001), plasma Ca and plasma $PGE_2$ levels ($r$=0.635, $p$<0.05), and plasma Ca and plasma TRAP activities ($r$=0.584, $p$<0.05) from the analysis using samples of both reproductive and non–reproductive stages. Taking these data into consideration, we suggested that $PGE_2$ acts on osteoclasts and increases plasma Ca as a result of osteoclastic bone resorption, and we concluded that $PGE_2$ is an important hormone in Ca metabolism during fish reproduction.

**Key words**: goldfish, plasma Ca, Prostaglandin $E_2$, reproduction, TRAP

## INTRODUCTION

Prostaglandin $E_2$ ($PGE_2$) functions to bone metabolism and is an important hormone in bone and promoter of osteoclastogenesis (Kaji *et al*., 1996; Gardner, 2007; Kaneko *et al*., 2007). The bone–resorbing activity of mature osteoclasts in osteoblast–containing mouse bone cell cultures was increased by $PGE_2$, although it did not affect osteoclast–like cell formation in osteoblast–free mouse spleen cell cultures (Kaji *et al*., 1996). Therefore, we focused on fish scales that coexist with both osteoclasts and osteoblasts (Bereiter–Hahn and Zylberberg, 1993; Suzuki *et al*., 2000; Yoshikubo *et al*., 2005; Suzuki *et al*., 2007; Suzuki *et al*., 2008; Suzuki *et al*., 2011; Yano *et al*., 2013). Using the goldfish scale *in vitro* assay system, we recently demonstrated that $PGE_2$ acts on osteoblasts and then increases the osteoclastic activity in the scales of goldfish as it does in the bone of mammals (Omori *et al*., 2012). In addition, the intraperitoneal injection of $PGE_2$ into goldfish induced hypercalcemia (Omori *et al*., 2012).

In the reproductive period, the plasma calcium (Ca) level in female teleosts increases remarkably (Watts *et al*., 1975; Yamauchi *et al*., 1978; Norberg *et al*., 1989; Suzuki *et al*., 2004). This Ca is bound to vitellogenin, which is a major component of egg protein and the calcium–binding protein (Tinsley, 1985; Kwon *et al*., 1993). In this period, $PGE_2$ synthesized in the ovaries functions to cause ovulation in fish (for a review, see Takahashi *et al*., 2013). As van Anholt *et al*. (2003) reported that $PGE_2$ in the blood may serve some physiological roles in fish, $PGE_2$ secreted from the ovaries might influence plasma Ca in fish. However, there has been no reported relationship between $PGE_2$ and Ca metabolism during the fish reproductive period.

To clarify the Ca metabolism in fish reproduction, we examined plasma $PGE_2$ and Ca levels and measured tartrate–resistant acid phosphatase (TRAP) activities as an indicator of osteoclastic activity in goldfish.

We concluded that $PGE_2$ is an important hormone in Ca metabolism during fish reproduction.

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## MATERIALS AND METHODS

### Animals

Female goldfish (n=14, 49.16±3.77 g) were purchased from a commercial source (Higashikawa Fish Farm, Yamatokoriyama, Japan) and used in the present study. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

### Measurement of plasma $PGE_2$, Ca, and TRAP levels of female goldfish in reproductive and non–reproductive stages

Goldfish in both the reproductive (March) (n=8) and non–reproductive (August) (n=6) stages were anesthetized with ethyl 3–aminobenzoate methanesulfonic acid salt (Sigma–Aldrich Inc., MO, USA). After weighing, the gonad somatic index (GSI) (%) was calculated. A blood sample was then collected from the dorsal aorta using a heparinized syringe. After centrifugation at 15,000 rpm for 3 mm, the plasma was immediately frozen and kept at –80°C until use. The plasma total Ca (mg/100 ml) and $PGE_2$ (pg/ml) levels were determined using specific assay kits (Ca: Calcium E test; $PGE_2$: $PGE_2$–ELISA kit, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

The plasma TRAP level was measured using 2 $\mu$l of plasma from each goldfish. TRAP activities were measured using an acid tartrate buffer (a 20 mM tartrate in a 0.1 M sodium acetate buffer (pH 5.3)). An aliquot of 100 $\mu$l of 20 mM para–nitrophenyl phosphate in an acid tartrate buffer was added to each well in a 96–well microplate. This plate was then incubated at 20°C for 30 min while being shaken. After incubation, the reaction was stopped by adding 50 $\mu$l of a 3 N NaOH–20 mM EDTA solution, and the absorbance was then measured at 405 nm. The absorbance was converted into the amount of produced para–nitrophenol (pNP) using a standard curve for pNP.

### Statistical analysis

All results are expressed as the means ± SE. Statistical significance was assessed by Student's $t$–test. Simple correlation coefficients were calculated to assess the relationship among GSI values, plasma $PGE_2$ levels, plasma Ca levels, and plasma TRAP activities. The statistical significance of the correlation was evaluated using the method of Snedecor and Cochran (1980). In all cases, the selected significance level was $p<0.05$.

## RESULTS

### Changes in GSI, plasma $PGE_2$ levels, Ca levels, and TRAP activities of female goldfish in reproductive and non–reproductive stages

There was a significant difference in the values of GSI between goldfish in March and August (Fig. 1). In addition, the plasma $PGE_2$ levels, Ca levels, and TRAP activities of female goldfish in March were significantly higher than those in August (Figs. 2, 3, and 4).

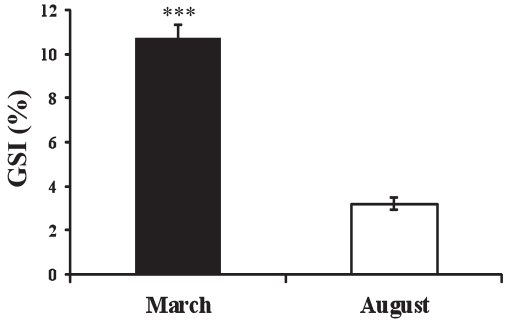


**Fig. 1.** GSI values of female goldfish in the reproductive (March) and non–reproductive (August) stages. *** indicates a statistically significant difference at $p<0.001$ in the values of the reproductive and non–reproductive stages.

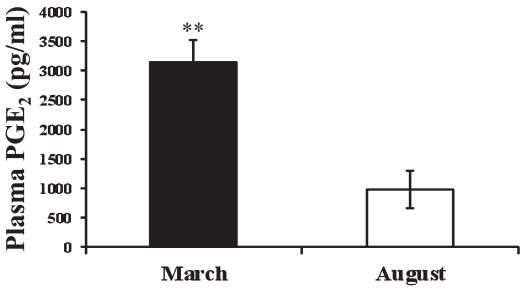


**Fig. 2.** Plasma $PGE_2$ values of female goldfish in the reproductive (March) and non–reproductive (August) stages. ** indicates a statistically significant difference at $p<0.01$ in the values of the reproductive and non–reproductive stages.

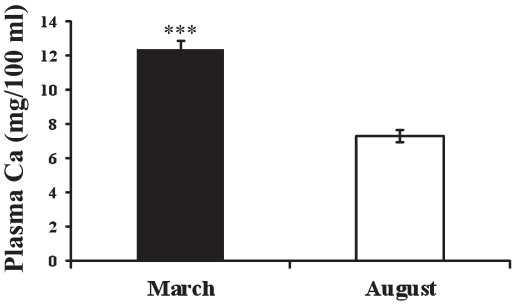


**Fig. 3.** Plasma Ca values of female goldfish in the reproductive (March) and non–reproductive (August) stages. *** indicates a statistically significant difference at $p<0.001$ in the values of the reproductive and non–reproductive stages.

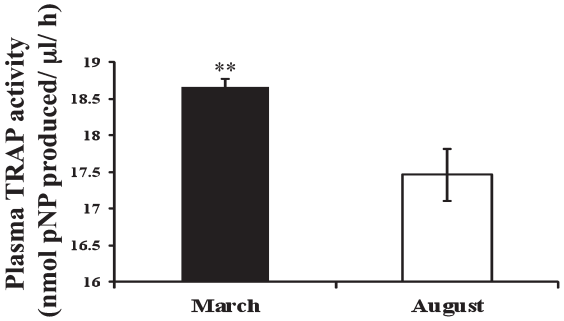


**Fig. 4.** Plasma TRAP activity (nmol pNP produced/$\mu$l/h) values of female goldfish in the reproductive (March) and non–reproductive (August) stages. ** indicates a statistically significant difference at $p<0.01$ in the values of the reproductive and non–reproductive stages.

**Correlation among GSI, plasma $PGE_2$ levels, Ca levels, and TRAP activities**

The results of correlation among GSI, plasma $PGE_2$ levels, Ca levels, and TRAP activities are indicated in Table 1. Significant positive correlations were recognized between GSI and plasma $PGE_2$ ($r$=0.790, $p$<0.001), GSI and plasma Ca ($r$=0.813, $p$<0.001), and GSI and plasma TRAP ($r$=0.631, $p$<0.05) from the analysis using samples of both reproductive and non–reproductive stages.

**Table 1.** Correlation among GSI, plasma Ca levels, $PGE_2$ levels, and TRAP activities (n=14).

| | $r$ values | $p$ values |
|---|---|---|
| GSI vs Plasma $PGE_2$ | $r$=0.790 | $p$=0.0007 |
| GSI vs Plasma Ca | $r$=0.813 | $p$=0.0004 |
| GSI vs Plasma TRAP | $r$=0.631 | $p$=0.015 |
| Plasma Ca vs Plasma $PG_{E2}$ | $r$=0.635 | $p$=0.014 |
| Plasma Ca vs Plasma TRAP | $r$=0.584 | $p$=0.028 |
| Plasma $PGE_2$ vs Plasma TRAP | $r$=0.514 | $p$=0.058 |

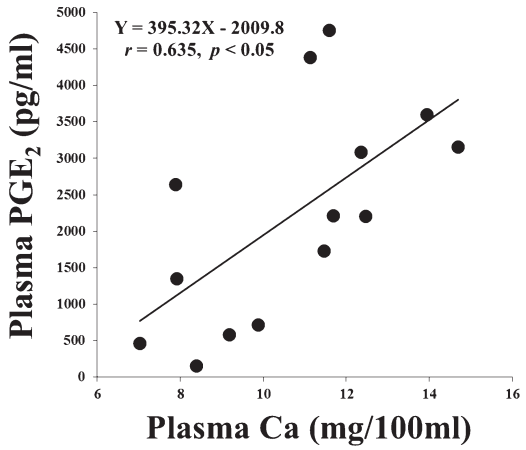


**Fig. 5.** Relationship between $PGE_2$ (pg/ml) and Ca (mg/100 ml) in the plasma of goldfish in the reproductive (March) and non–reproductive (August) stages.

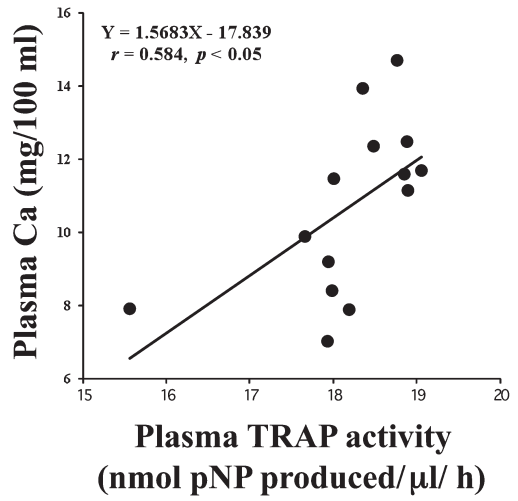


**Fig. 6.** Relationship between Ca (mg/100 ml) and TRAP activities (nmol pNP produced/μl/h) in the plasma of goldfish in the reproductive (March) and non–reproductive (August) stages.

As a result of having paid attention to the relations with plasma Ca, we discovered a significant positive relationship between plasma Ca and $PGE_2$ ($r$=0.635, $p$<0.05) (Fig. 5), and between plasma Ca and TRAP ($r$=0.584, $p$<0.05) (Fig. 6).

As $PGE_2$ levels increased, plasma TRAP activities tended to rise ($r$=0.514, $p$=0.058).

## DISCUSSION

The present study is the first to demonstrate that $PGE_2$ is related to Ca metabolism in fish reproduction. Corresponding to increased GSI, plasma $PGE_2$ levels, Ca levels, and TRAP activities rose. In addition, significant correlations between plasma Ca and $PGE_2$ and between Ca and TRAP were observed. Because TRAP is known as an osteoclast–specific marker (for a review, see Vaes, 1988), the increased $PGE_2$ in the March fish activated osteoclasts and promoted osteoclastic bone resorption. As described in the introduction, we recently demonstrated that $PGE_2$ acts on osteoblasts and increases the osteoclastic activity in the scales of goldfish as it does in the bone of mammals (Omori *et al.*, 2012). In an *in vivo* experiment, furthermore, hypercalcemia was induced as a result of osteoclastic bone resorption after an intraperitoneal injection of $PGE_2$ into goldfish (Omori *et al.*, 2012). Taking these results into consideration together with the present study, we have concluded that $PGE_2$ acts as a calcemic hormone in fish reproduction.

In the present study, the highest correlation between GSI and plasma Ca was recognized. We think that several hormones, with the exception of $PGE_2$, are involved in Ca metabolism during fish reproduction. The candidate for this hypercalcemic hormone is estrogen. In female teleosts, estrogen enhances the synthesis of vitellogenin, which is a major component of egg protein and a Ca–binding protein (Tinsley, 1985; Kwon *et al.*, 1993). At the same time, estrogen promotes Ca resorption from the scales by activating osteoclasts (Persson *et al.*, 1995; Suzuki *et al.*, 2000; Suzuki and Hattori, 2003; Suzuki *et al.*, 2009). Consequently, plasma vitellogenin and Ca levels increase corresponding to the increase in estrogen level (Norberg *et al.*, 1989). $PGE_2$ is closely related to ovulation (late stage of fish reproduction) (for a review, see Takahashi *et al.*, 2013), suggesting that in the early stage of fish reproduction, estrogen acts as a hypercalcemic hormone, and then $PGE_2$ plays roles in both ovulation and Ca metabolism.

On the other hand, we previously demonstrated that a hypocalcemic hormone, calcitonin, acts on scales and inhibits osteoclastic activity using an *in vitro* scale assay system with goldfish (Suzuki *et al.*, 2000). As estrogen activates osteoclasts in some teleosts in both *in vivo* and *in vitro* experiments (Persson *et al.*, 1995; Suzuki *et al.*, 2000; Suzuki and Hattori, 2002; Suzuki and Hattori, 2003; Suzuki *et al.*, 2009), a counteraction may

exist between calcitonin and estrogen in osteoclasts of the scale. Using the *in vitro* scale assay system, the increased osteoclastic activity with estrogen was actually suppressed by calcitonin in goldfish (Suzuki *et al*., 2000). Furthermore, our previous study demonstrated the interaction between calcitonin and estrogen. In the ultimobranchial gland, which is the secretion organ of calcitonin, estrogen receptors were detected by estrogen–specific binding assay and immunohistochemical analysis in goldfish (Suzuki *et al*., 2004). Also, three types of estrogen receptors were detected in the ultimobranchial gland of goldfish (Suzuki *et al*., 2004). Moreover, just after injecting estrogen into goldfish, plasma calcitonin level increased before the rise of plasma Ca (Suzuki *et al*., 2004). Considering from our present data, we strongly suggested that $PGE_2$ affects other calcemic hormones in fish reproduction. Thus, in the future, we will examine the interaction among calcemic hormones, such as $PGE_2$, calcitonin, and estrogen, and elucidate the mechanism of teleost bone metabolism during the reproductive stages.

## ACKNOWLEDGMENTS

This study was supported in part by grants to N.S. (Grant–in–Aid for Scientific Research [C] No. 24620004 by JSPS), to A.H. (Grant–in–Aid for Scientific Research [C] No. 24570068 by JSPS), to T. S. (Grant–in–Aid for Young Scientists [B] No. 40378568 by JSPS), to H.M. (Grant–in–Aid for Scientific Research [C] No. 23592727 by JSPS), and to K.H. (Grant–in–Aid for Scientific Research for Exploratory Research No.24651044 by JSPS).

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